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Assimilation, Transport, and Distribution of Molecules in Insects from Natural and Artificial Diets

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Insect Rearing and Progress in Insect Control

Rearing systems have provided research and development laboratories with the bases for numerous bioassays, usually in the form of raw materials—the insects—to be used in vivo or in vitro. Modified rearing systems themselves are sometimes used for in vivo bioassays. In any case, insect rearing has been the foundation for the discovery and development of most conventional insecticides and biological control agents. Now, biotechnology is offering new tools for the discovery and implementation of insect control methods. A strong argument can be made that biosynthetic compounds and engineered plants, microorganisms, and other agents will gradually supplement and supplant our diminishing arsenal of synthetic organic insecticides (Table 1) (Meeusen & Warren 1989). Although all the examples in Table 1 are proteinaceous, and therefore macromolecules, simple organic compounds may also eventually be engineered into a system by introducing genes coding for enzymes that either synthesize simple compounds de novo or modify existing compounds. Rearing systems will play a vital role in these developments and will reflect changing ideas about the physical, chemical, and biological interfaces between insect and control agent.

As novel control agents are developed, emphasis in research will change from organic compounds, synthesized and formulated by chemists, to macromolecular or organic natural products, synthesized by organisms and usually formulated as components of those same organisms. Modes of penetration, translocation, and effects at target sites will differ from those of conventional insecticides. Digestion, penetration, absorption, and transport

Table 1. Examples of insecticidal or insect-bioregulatory molecules with potential for use as insect control agents, or components of such agents, through genetic engineering

Molecule	Source	Reference
δ-Endotoxin	*Bacillus thuringiensis*	Obukowicz et al. 1986a,b Ahmad et al. 1989
Cowpea Trypsin Inhibitor	*Vigna unguiculata*	Hilder et al. 1987
Proteinase Inhibitors I/II	*Solanaceae*	Johnson et al. 1989
Juvenile Hormone Esterase	*Heliothis virescens*	Hammock et al. 1990

through the hemolymph from the digestive tract to target organs will become especially important in making control agents effective. Conventional insecticides generally enter an insect through the cuticle and must be able to readily penetrate the cuticle to be effective. (The chemical and physiological determinants of penetration are reviewed by Gilby 1984 and Welling & Paterson 1985.) Topical application has been very useful for testing the toxicities of cuticle-penetrating substances. With the advent of biologically engineered control agents, the insect alimentary canal will become the prime site for penetration of novel agents, limiting the value of topical assays. Instead, future assays for biologically derived agents will be based on dietary systems.

The insect diets used in rearing and assay are central to discovering, developing, and enhancing the efficacy of novel agents, and to understanding their natural roles. However, the complications of dietary interactions among allelochemicals and nutrients in artificial and natural diets sometimes dramatically affect nutrient utilization (Reese 1979). Conversely, nutrients can affect the efficacy of allelochemicals. Understanding the interactions between nutrients and allelochemicals or engineered agents in the context of digestion, assimilation, translocation, and mode of action can save effort and increase rates of product discovery and development.

This article will briefly summarize the complex assimilative and translocative processes in insects and note the most important characteristics of dietary components to consider in the rearing and use of insects for assay of control agents. Putative roles of hemolymph proteins in the processes of absorption and transport will also be discussed. As background to these physiological processes, reviews from several fields of study will be cited throughout this work.

In the most prominent examples of engineered control agents, genes for *Bacillus thuringiensis (B.t.)*, δ-endotoxins are being successfully incorporated into a variety of microbial and plant species (Meeusen & Warren 1989). After ingestion, the δ-endotoxin proteins, in the form of isolated protein or genetically incorporated into host organisms, act at the insect midgut. The endotoxins enter and affect the insect only through the digestive system. They are activated by physical and biochemical factors in the midgut and act directly on midgut cells. Cellular penetration may not even be necessary for toxicity, since binding of the endotoxins to high affinity sites occurs at the brush border membrane (Hilder et al. 1987). Despite distinct chemical contrasts between the macromolecular endotoxins and small compounds, the δ-endotoxins can serve to contrast conventional and engineered insect control agents.

Formulations: An Analogy

Toxicologically, the transformed microbial or plant host of the δ-endotoxin gene serves two roles, each of which can be compared to an analogous role in the manufacture of a conventional synthetic insecticide (Fig. 1):

1. The host is the actual producer of the δ-endotoxin, biosynthesizing it from the genetic template. The synthetic chemist fulfills this role in the manufacture of conventional insecticides.
2. The transformed host serves as the formulation for delivery of the δ-endotoxin to the insect. Formulation of a conventional insecticide includes carriers, wetting agents, synergists, etc., in addition to a small percentage of active agent, and can turn an isolated compound of moderate activity into a potent mixture through effects on the external and internal physiology of the target insect. The toxicity and toxicokinetics of a formulated insecticide may therefore differ considerably from those of the isolated insecticide.

Since the δ-endotoxin in an engineered system is ingested as an integral component of the host organism, that formulation complicates comparisons between the isolated toxin administered in a feeding assay and the host endotoxin

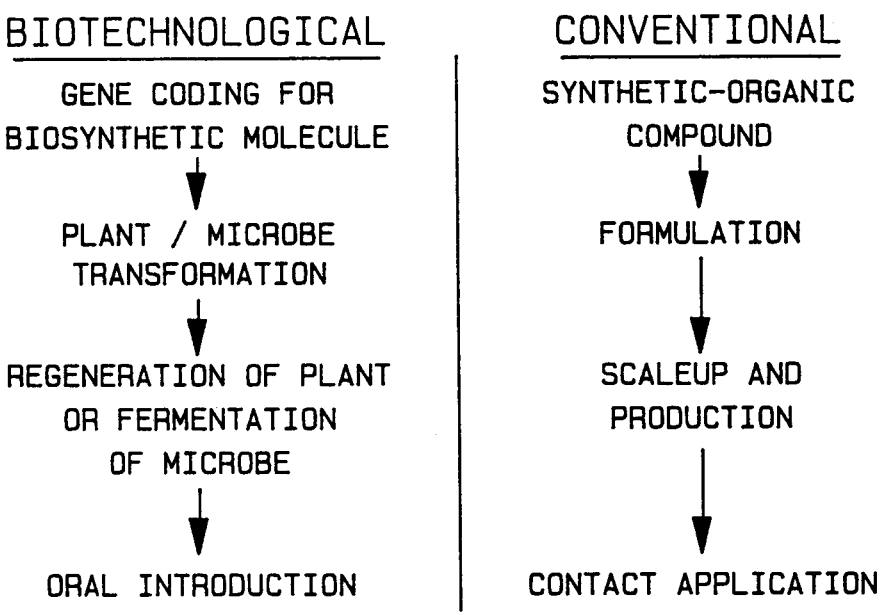

Fig. 1. Analogy between steps in derivation of biotechnological and synthetic insect control agents.

system administered in the greenhouse or field. In the engineered system, calculations of basic parameters such as absolute dose and rate of exposure to the toxin are complicated by levels of expression of the toxin and rate of feeding of an insect on the host plant. Even harder to calculate or predict, however, are the effects of a host plant's biochemistry on efficacy of an engineered effector.

To compare results from laboratory assays with applications in the field, an enhanced understanding of insect digestive physiology and biochemistry will be required. Among the processes to be understood, our knowledge is limited regarding stability of an agent in the gut, penetration through (or effect upon) the midgut epithelium, and transport to active sites via the hemolymph. However, we should be able to derive operative concepts from a parallel understanding of synthetic chemical control agents and their formulations.

Artificial Versus Natural Diets

Artificial diets are simplified, optimized versions of natural diets. Simplification of the natural milieu leaves out numerous compounds and their

polymers that would otherwise be absorbed to both the detriment and benefit of the insect. Optimization yields a diet that aims to achieve maximal growth rate and final size of the insect; optimization thus refers to conditions that "benefit" the insect within a restricted, subjective, utilitarian definition of the term.

While optimized diets yield maximal growth and production of an insect species, they do little for comparing various biochemical control agents. Many studies on effects of natural products are conducted with artificial rearing systems. Isolated natural products or mixtures of them are combined with artificial diet or layered over the diet, forcing ingestion by the insect under study. Other studies compare insects fed an artificial diet with those fed a natural diet. In either case, the comparison is limited by the complexities of and differences among the dietary matrices. When examining the effects of a substance or mixture on an insect, many factors 1) influence digestion, absorption, transport, and toxicities, and 2) complicate comparisons between control and experimental groups and between laboratory and greenhouse or field experiments. The following factors can distinguish activities in artificial diets from those in natural or genetically engineered diets.

Active Biotic Factors. Microbes or active biochemical factors such as enzymes that are usually present in natural systems will rarely be present in artificial systems. Such factors may alter the apparent chemical composition of an engineered agent once it is ingested. One example of this involves midgut symbionts. In aphids and certain other insects, destruction of symbionts by antibiotics can result in depletion of necessary products such as sterols that are synthesized solely by the symbionts, resulting in inhibition of growth, development, and survival (Mittler 1971a, 1971b). Since antibiotics and antiseptics are commonly used in artificial diets, their effects on putative control agents must be considered.

Complexation of Active Components. Within the matrix of either natural or artificial diet, compounds or macromolecules may complex with other compounds or macromolecules. Some may become less available for digestion and absorption, while others may be more readily absorbed through cooperative effects. The tannins and α-tomatine are examples of the former. Tannins bind and precipitate proteins, and their ingestion may result in significant decrease in protein digestibility (Feeny 1968, Reese 1979, Duffey 1980). Binding of activated δ-endotoxin by high concentrations of tannins in some plant species could prevent binding of the toxin to midgut cells, decreasing its activity; binding of proteolytic enzymes by tannins could inhibit proteolytic activation of the δ-endotoxin.

α-Tomatine, an alkaloid in tomato plants, apparently exerts toxicity against insect herbivores and their parasites by binding ß-sterols. α-Tomatine might be

useful as an allelochemical component engineered or bred into crops if it were not for its potential impact on useful parasites, parasitoids, and predators (Campbell & Duffey 1979). Its toxicity can be alleviated by high dietary content of ß-sterols (Campbell & Duffey 1981), possibly complicating interpretation of studies in which sterol content varies between natural and artificial diets.

Changes in Midgut Environment. The biochemical and physical environments of the digestive system may be changed appreciably by the presence, absence, or quantity of a compound or mixture of compounds. With ingestion, induced or introduced changes in the gut environment may result in changes in pH, reducing potential, conductivity of specific ions, etc., or in changes in activities of many enzymes such as proteases or mixed function oxidases (MFOs) in the midgut. MFOs oxidize toxic compounds, reducing their activity and making them more excretable. Some secondary plant products significantly induce MFO activity in the insect midgut and can result in decreased toxicity of plant-derived allelochemicals, such as nicotine (Brattsten & Wilkinson 1977).

Internal Sensitivity to Active Agents. Coabsorption of dietary compounds with an active agent may alter the internal response or responsiveness of an insect at sites targeted by the active agent. This may occur directly, through synergism or antagonism toward the agent at the target site, or indirectly, through induction or repression of specific cellular systems such as membrane receptors. Synthetic organic insecticide formulations often include synergists and carriers.

The most common synergists, inhibitors of mixed function oxidases, increase the susceptibility of an insect to an active compound by inhibiting the enzymes that metabolize and thus inactivate that compound (Brattsten 1979). Inadvertently, some solvents used in insecticide formulation may do the opposite, inducing enzyme synthesis and effectively increasing resistance of the target insect (Brattsten et al. 1977). Applebaum (1985) gives examples of many naturally occurring substances that inhibit the digestive process, especially regarding proteolytic activity.

The Roles of Absorptive and Transport Systems

The need to understand the processes of digestion, absorption, and transport of compounds (as summarized in Fig. 2) in the insect should now be manifest. Although processes contributing to the removal of compounds from the insect (e.g., enzymatic metabolism) have been well studied, processes involved in addition of compounds to the system (e.g., absorption, transport, and binding)

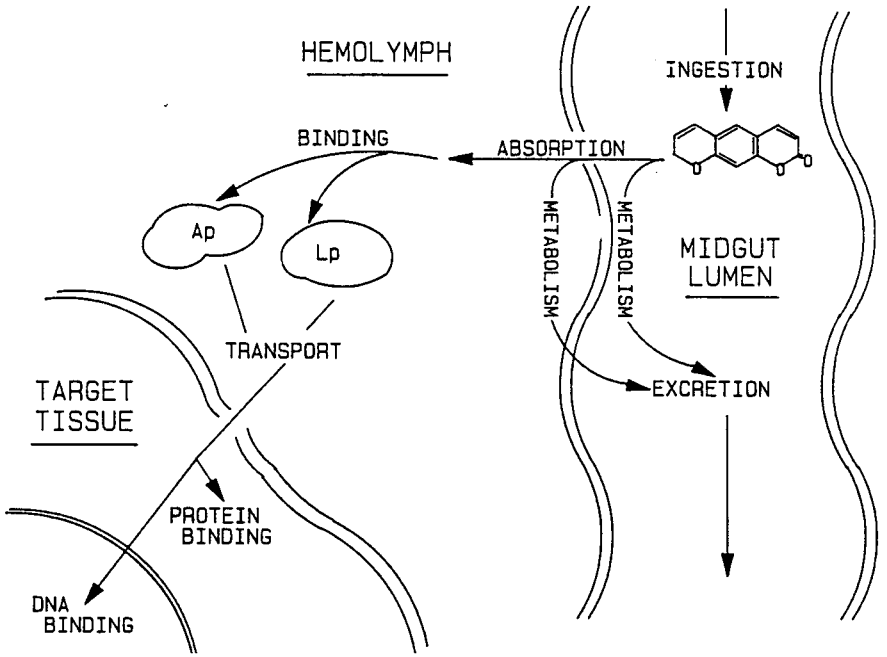

Fig. 2. Physiological processes involved in adding to or removing from a compound's availability to target sites.

are not as well understood. The following generalizations about these processes can aid in understanding, predicting, and comparing effects of compounds in rearing, assay, and field systems.

Digestion

Following ingestion, the process of digestion exerts the initial influence on absorption of a compound. Digestion involves a large number of enzyme-catalyzed biochemical reactions: hydrolysis of proteins to peptides and peptides to amino acids; breakdown of some complex carbohydrates (generally excluding cellulose, except in termites) to mono- or di-saccharides, and of polynucleotides (DNA, RNA) to purines and pyrimidines; and derivatization of low molecular weight compounds. (See Applebaum 1985 for descriptions of enzyme classes and the digestive process.) These processes usually result in products of increased

polarity (Brattsten 1979). Digestion therefore results in decreasing concentrations of macromolecules and increasing concentrations (and thus rates of absorption) of low molecular weight compounds.

Though digestion can result in either activation or inactivation of compounds, it is usually thought of as an inactivating process. Mixed function oxidases, glutathione transferase, and other enzymes act to increase polarity and excretability of numerous compounds at the midgut epithelium; proteolytic enzymes hydrolyze and inactivate protein toxins in the midgut lumen. However, digestion can also activate toxins, as with the protease- and pH-activated *B.t.* δ-endotoxin.

Digestive tissues and processes can be direct targets of agonists and antagonists. Proteolytic inhibitors are widespread in plants and may contribute to resistance against insect pests in many species (Ryan 1979, Gordon 1968). They usually act by deactivating the catalytic site or sites of a proteolytic enzyme and are among the agents being actively explored for use in biologically engineered plant defenses (Meeusen & Warren 1989, Haunerland & Bowers 1986). In an engineered system, the natural presence of such inhibitors in host plants might decrease efficacy of an incorporated protein such as *B.t.* δ-endotoxin by preventing necessary proteolytic activation. Rearing and assay systems, lacking the inhibitors, would not detect such a problem, which would appear in final stages of testing. Alternatively, protease inhibitors may synergize some proteinaceous agents by decreasing their digestion in the midgut.

Absorption

Two physiochemical factors have an immediate impact upon absorption rate: the size of a compound or molecule, and the polarity or lipophilicity of lower molecular weight compounds. The gut epithelium, and to a lesser extent the peritrophic membrane (if present in the species or stage of insect in question), act as molecular sieves. Above a certain molecular weight, penetration from lumen through the epithelium into the hemocoel is impossible, prohibiting passage of macromolecules such as complex carbohydrates, proteins, large nucleic acids, and other polymers.

Absorption of lower molecular weight compounds occurs through passive diffusion of apolar (lipophilic) compounds and some ions, through facilitated diffusion aided by shuttle proteins in membranes, or through active transport of compounds by energy-driven processes. The lipid-protein matrix of epithelial cell membranes inhibits penetration by polar compounds, except through facilitated diffusion or active transport by membrane proteins. Once a polar compound crosses the epithelial membrane, it is readily dissolved in the aqueous phase and taken into circulation. On the other hand, lipophilic compounds tend to diffuse readily into and across cell membranes, but their uptake into circula-

tion is limited by poor solubility in the aqueous phase of the hemolymph. Despite this fact, most effective insect toxins, both synthetic and natural, are moderately to highly apolar. These concepts sparked spirited debates as to whether toxic doses of insecticide are absorbed by vertical diffusion through cuticle into hemolymph or by horizontal diffusion into the spiracles and along tracheae to target organs. Though the debate was not conclusively settled, popular opinion supports vertical diffusion and transport to target organs through hemolymph (see Gilby 1984 or Welling & Paterson 1985 for complete discussions).

Transport

The apparent paradox of the high toxicities of insoluble apolar compounds may be resolved by realizing the biophysical nature of hemolymph: although an aqueous medium is not conducive to transport of apolar compounds, hemolymph is not purely aqueous. It contains high concentrations of proteins, and proteins are amphophilic ("loving both sides") macromolecules, i.e., they are compatible with both lipid and aqueous phases. As alluded to by Campbell and Duffey (1981), transport of lipophilic compounds by mammalian proteins is well known, but analogous modes of transport in insects are more obscure. Hemolymph proteins that bound insecticides in vivo and in vitro were first observed in the mid-1970s (Welling & Paterson 1985; Shapiro et al. 1988a), but specific proteins were not identified until 1984-1986.

The best known insect transport proteins are the lipophorins (Shapiro et al. 1988a). Lipophorins are lipoproteins, or spherical fluid particles of lipid and protein found in virtually all species of insects examined to date. Circulating freely in hemolymph, they absorb lipids from midgut and release them into fat body for storage and absorb lipid from fat body for delivery to sites of utilization. Firm evidence from several species shows lipophorins to function as lipid shuttles (see Chino et al. 1981 and Tsuchida & Wells 1988). Their protein moieties recirculate, while lipid components are transported unidirectionally. Insecticides are also bound by insect lipoproteins (Shapiro et al. 1988a), identified recently as lipophorins (Kawooya et al. 1985).

Another class of hemolymph protein, the arylphorins, has also recently been shown to bind insecticides when mixed in vitro with hemolymph from *Heliothis zea* (Haunerland & Bowers 1986). Arylphorins are known as amino acid storage proteins, thought to act as a sink for amino acids utilized in cuticle synthesis during metamorphosis. Binding of a range of insecticides by an arylphorin from *Heliothis zea* was a novel discovery. Perhaps of more consequence was that the proportion of a compound bound to arylphorin versus lipophorin was dependent upon the partition coefficient of the compound, representing its lipophilicity. Another example of xenobiotic binding to hemo-

lymph proteins has recently been discovered and described in vivo and in vitro, although the binding protein has yet to be identified with any known class of insect protein. In the root weevil larva *Diaprepes abbreviatus*, a model fluorescent compound (7-amino-3-phenyl coumarin, or coumarin-10) was absorbed from a semidefined diet (Shapiro et al. 1988b), and from a force-fed mixture in oil (Shapiro 1989), into hemolymph. A large protein of 480,000 molecular weight, present at concentrations approaching 100 mg/ml, bound 95% of the coumarin-10 found in hemolymph; lipophorin bound 5%. A dissociation constant of 1.5×10^{-6} M was determined in vitro.

These few examples of xenobiotic binding by proteins and lipoproteins in hemolymph supply a link between absorption and target site interaction (Fig. 2). The kinetics of toxicity are critical: A toxic compound, whether an insecticide (Welling & Paterson 1985) or phytochemical (Duffey et al. 1978) must interact with a target site at a concentration high enough to produce the desired effect. An increased rate of transfer from the midgut serosa into hemolymph and subsequent transport to target sites may increase availability of the compound in the critical concentration at the target site.

Binding of apolar compounds may involve proteins in hemolymph other than the arylphorins and lipophorins. Even regarding lipophorins, binding and transport of compounds other than the native lipids are poorly described, and the kinetics of uptake and transport are virtually unknown. The roles of hemolymph proteins relative to intoxication versus detoxification are also unknown. Knowledge of their roles in the uptake and transport processes can aid in the discovery and increased efficacy of control agents.

Conclusion and Summary

Although assimilative processes are complex, awareness of the biochemistry in a natural or engineered system modeled by an assay system can highlight critical relationships among components, and problems may be alleviated during system design. For example, if one is testing candidates for a proteinaceous toxin to engineer into a plant variety that contains some known proteolytic enzymes, similar enzymes can be included in a diet assay system. Comparing conditions for assimilation of natural versus artificial diets can therefore reveal pitfalls between the processes of discovery and implementation of bioregulatory agents, or between observations in a laboratory versus a field environment. Both quantitative (e.g., significant differences in LD_{50}) and qualitative (e.g., toxicity versus nontoxicity) differences in activity of an agent may be confusing to an investigator.

To aid in the design of dietary systems, additional knowledge of assimilative processes is necessary. The basic enzyme classes and their roles in digestion are

well defined, and selected enzyme activities in an insect can be readily studied. However, absorption is more difficult to study. General principles are given for absorption of specific biochemical classes (Turunen 1985), but the range of compounds to be studied is much too diverse and methods too painstaking for thorough study in any one insect. Although general principles for transport of polar compounds are clear, those for transport and final disposition of lipophilic compounds are still obscure and deserve further attention.

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